

Variability in the Antioxidant Activity of Dietary Supplements from Pomegranate, Milk Thistle, Green Tea, Grape Seed, Goji, and Acai: Effects of *In Vitro* Digestion

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ABSTRACT: The antioxidant activity (AA) of fruits and vegetables has been thoroughly investigated but less is known about the AA of dietary supplements (DS). We therefore assessed the AA of three to five DS each from pomegranate, milk thistle, green tea, grapes, goji, and acai using four widely used standard methods. The secondary objective was to determine the effects of *in vitro* digestion on their AA. The AA of the DS prior to digestion ranked as follows: pomegranate > resveratrol > green tea > grape seed > milk thistle and very low in goji and acai with significant group variability in AA. The AA after *in vitro* simulated digestion of the mouth, stomach, and small intestine compared to undigested supplement was decreased for green tea and grape seed but increased for pomegranate, resveratrol, milk thistle, goji, and acai to various extents. Although polyphenols provide the major antioxidant potency of the tested supplements, our observations indicate that digestion may alter antioxidant properties depending in part on the variations in polyphenol content.

KEYWORDS: pomegranate, antioxidant activity, *in vitro* digestion, dietary supplements, green tea, milk thistle, acai, goji, resveratrol, grape seed

INTRODUCTION

Over half of U.S. adults consume at least one dietary supplement (DS) per day as estimated by data from the National Health and Nutrition Examination Survey (NHANES) 1999–2002.¹ According to Yang et al.,² DS provide 25% of dietary total antioxidant capacity in U.S. adults. Polyphenolic phytochemicals are the most frequently occurring phytochemicals in DS with *in vitro* antioxidant properties.³ On the basis of an evaluation of the NHANES 2001–2002 data, flavonoid and proanthocyanidin intake contributes 56% of total antioxidants in the diet.² Although polyphenols are principally ingested in the diet, commercially available polyphenolic supplements are numerous and commonly used and may contribute to the antioxidant intake. Typical DS that contain antioxidant polyphenols are derived from pomegranate fruit (*Punica granatum* L.), green tea (*Camellia sinensis*), and grapes (*Vitis vinifera*). Other DS such as milk thistle (*Silybum marianum*) extract, acai (*Euterpe oleracea*), and goji berries (*Lycium barbarum* L.) are commonly consumed for their antioxidant activity (AA) but contain very different phytochemical structures (e.g., stilbenes for resveratrol), all of which have some AA.

The main phytochemicals held responsible for AA vary considerably in these DS. Pomegranate fruit contains ellagitannins, gallotannins, ellagic acid, and smaller amounts of flavonoids and anthocyanins.^{4–6} Green tea extracts contain polyphenols including (–)-epicatechin, (–)-epigallocatechin, and their gallate derivatives (–)-epicatechin-3-gallate and (–)-epigallocatechin-3-gallate, which are distinguished by their potent antioxidant and anti-inflammatory properties,

offering cardiovascular and neural protection, and for their effects on fat oxidation.⁷ Proanthocyanidins in grape seed extract are the main polyphenolic ingredients responsible for its antioxidant, anti-inflammatory, and antimicrobial effects.^{7,8} Resveratrol (3,5,4'-trihydroxystilbene) is a stilbene polyphenol that exerts anti-inflammatory, antioxidant, and estrogenic effects and anticancer properties.⁹ Although advertised as a grape extract, it is commonly extracted from the root of Japanese knotwood and occurs as a phytoalexin on the skin of grapes grown in humid conditions, where they are susceptible to fungal infection. The fruit of the milk thistle plant, with its active ingredient and flavonoid complex, silymarin, has been used to promote liver health. Silybin, a flavonolignan, represents about 50–70% of the silymarin extract, among other flavonolignans and flavonoids. Silybin and the rest of the silymarin constituents have demonstrated liver-protective properties under experimental conditions due to their antioxidant and anti-inflammatory properties.¹⁰ In recent years, two berry species rich in polyphenolic phytochemicals, acai berry (Amazonian palm berry) and goji berry, have been widely promoted as having antioxidant properties attributed to their primary active ingredients, β -carotene, β -cryptoxanthin, zeaxanthin, and polysaccharides in goji berry fruit¹¹ and anthocyanins and other polyphenols in acai berry.^{12–15}

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It was the objective of the present study to assess the variability of composition and AA of three to five commercially available DS from pomegranate, green tea, grape seed, milk thistle, resveratrol, acai, and goji berry using four antioxidant assays. The oxygen radical absorbing capacity (ORAC) hydrophilic and lipophilic, which measures a hydrogen atom transfer reaction, was used in addition to assays measuring electron transfer/reducing capacities such as the Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), and free radical scavenging properties by the diphenyl-1-picrylhydrazyl (DPPH) radical.

In a second phase, we determined the effect of digestion on the AA of these popular DS because we were interested in whether antioxidant activity would survive the digestive processes. Although in vivo feeding or supplementation studies in animals or humans provide the ultimate tests, in vitro digestion models simulating digestive pH and enzymatic exposure provide a practical alternative for investigating the impact of digestion on DS antioxidant properties.¹⁸ The DS were treated under in vitro simulated gastrointestinal conditions, and the AA was analyzed using the TEAC assay. The TEAC assay was chosen from among the four antioxidant assays used in the first phase of our studies because TEAC exhibited the highest correlation with the sum of all antioxidant assays. It was also the most reproducible among the methods used.

MATERIALS AND METHODS

Chemicals and Reagents. All solvents were purchased from Fisher Scientific Co. (Tustin, CA, USA). 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), manganese dioxide, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ferrous chloride, fluorescein sodium salt, and (2,4,6-tris(2-pyridyl)-s-triazine, 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Randomly methylated β -cyclodextrin was purchased from CTD Holdings (Alachua, FL, USA).

Dietary Supplements. A total of 25 antioxidant DS from 7 different categories, acai berry ($n = 3$, A1–A3), goji berry ($n = 3$, G1–G3), grape seed ($n = 3$, GS1–GS3), green tea ($n = 4$, GT1–GT4), milk thistle ($n = 4$, MT1–MT4), pomegranate ($n = 5$ samples, PM1–PM5), and resveratrol ($n = 3$, Res1–Res3), were obtained on the basis of accessibility to the average consumer (purchased from retail stores, chain pharmacies, Internet, or mail order). The DS were delivered in the form of encapsulated powder ($n = 17$ samples), soft gel ($n = 4$), tablet ($n = 2$), or liquid ($n = 2$). The antioxidant activity of all DS was determined prior to the expiration date as stated on their packages. The main information found on the packaging label of all tested DS is listed in Table 1. The pomegranate extract DS tested were PomX (POM Wonderful LLC, Los Angeles, CA, USA), Healthy Pomegranate (Nature's Bounty, Bohemia, NY, USA), Full-Spectrum Pomegranate (Life Extension, Ft. Lauderdale FL, USA), Pomegranate 40:40 Complex (All Solar, Henderson, NV, USA), and Pomella (+PLUS) (Phytosensia, Indianapolis, IN, USA; and Pomella, Noblesville, IN, USA). The green tea extract DS tested were Green Tea Extract (Radiance, Encinitas, CA, USA), Green Tea Complex (Safeway Inc., Pleasanton, CA, USA), Standardized Extract Green Tea (Nature's Bounty), and Green Tea Standardized (Nature's Way, Lehi, UT, USA). The milk thistle extract DS used were Milk Thistle XTRA (Sundown Naturals, Boca Raton, FL, USA), Standardized Extract Milk Thistle (Nature's Bounty), Super Maximum Milk Thistle Extract (Vitamin Shoppe, North Bergen, NJ, USA), and Milk Thistle (Safeway, Pleasanton, CA, USA). The grape seed extract DS used were Grapeseed Extract (Radiance, Ronkonkoma, NY, USA), Masquelier's Tru-OPCs (Nature's Way), and ActiVin Grape Seed Extract (Paradise, Huntington Beach, CA, USA). The acai extract DS were Acai (Nature's Bounty), Acai Extract (Vitamin Shoppe), and Acai

Table 1. Labeling Information of All Dietary Supplements^a

ID	main ingredients
pomegranate	
PM1	pomegranate extract
PM2	pomegranate extract (40% ellagic acid)
PM3	pomegranate extract [30% punicalagins (120 mg)]; plus pomegranate blend [22% (30 mg) punicalic acid]
PM4	vitamin C; plus pomegranate extract (40% punicalagins/40% ellagic acid)
PM5	pomegranate extract [30% punicalagins (240 mg)]
green tea extract	
GT1	green tea extract (15% polyphenols, 95 mg in two capsules)
GT2	green tea extract (25% polyphenols, 8.5% EGCG, 5% caffeine), 790 mg; plus green tea powder (leaf, 210 mg)
GT3	green tea extract (15% polyphenols 95 mg in two capsules)
GT4	green tea extract (9% polyphenols) plus green tea (leaf)
resveratrol	
Res1	resveratrol, 100% <i>trans</i> -resveratrol, 175 mg; plus Japanese sophora flower bud extract, 74% rutin (quercetin rutinoside), 75 mg
Res2	250 mg of <i>trans</i> -resveratrol; plus quercetin (quercetin dihydrate, 100 mg)
Res3	red wine extract (30% polyphenols, 60 mg); plus red wine powder; plus Japanese knotweed (50% resveratrol 75 mg)
grape seed extract	
GS1	grapeseed extract (50% polyphenols 100 mg); plus citrus bioflavonoids 300 mg
GS2	grape seed extract (95% antioxidants; 65% oligomeric procyanidins (OPCs), 30% other active polyphenols
GS3	grape seed extract 100:1
milk thistle	
MT1	milk thistle extract (80% silymarin 192 mg)
MT2	milk thistle extract (80% silymarin 140 mg)
MT3	milk thistle extract (80% silymarin 240 mg)
MT4	milk thistle extract (80% silymarin 140 mg)
acai	
Acai1	acai extract
Acai2	acai berry 5:1 extract
Acai +GT	acai (20:1 extract equivalent to 1000 mg); plus green tea (4:1 extract equivalent to 500 mg)
goji	
Goji1	100% goji juice
Goji2	goji liquid concentrate
Goji3	goji berry extract (20% polysaccharides)

^aDS, dietary supplements; PM, pomegranate; GT, green tea; EGCG, (–)-epigallocatechin gallate; GS, grape seed; Res, resveratrol; MT, milk thistle.

with Green Tea (Radiance). Resveratrol extracts obtained were Resveratrol forte (Enzymatic Therapy, Green Bay, WI, USA), Reservic and Trans-Resveratrol (Vitamin Shoppe), and Resveratrol, Synergistic Formula (Nature's Way). Goji berry juice/extracts used were Goji Gold (Dynamic Health, Brooklyn, NY, USA), Goji 100 (Genesis Today, Austin, TX, USA), and Goji Berry (Ultra Laboratories, Carson, CA, USA).

Digestive Enzymes. All digestive enzymes were purchased from Sigma-Aldrich. All enzyme solutions were freshly prepared for each experiment to avoid potential decreases over time in enzyme activities. α -Amylase from *Aspergillus oryzae* (~30 units/mg) was dissolved in the phosphate buffer saline (PBS) solution (160 units/mL) that was preadjusted to pH 6.5. Porcine pepsin from gastric mucosa (≥ 250 units/mg solid) was dissolved at 40 mg/mL in 0.1 M HCl. Pancreatin from porcine pancreas was mixed with a porcine bile extract mixture at a constant ratio of 1:6 and dissolved in 0.1 M NaHCO₃. Pancreatin contains several enzymes, including amylase, trypsin, lipase, ribonuclease, and protease. Bile extract contains glycine and taurine conjugates of hyodeoxycholic acid and other bile salts.

In Vitro Digestion. The in vitro digestion was performed according to the methods described by Chohan et al.¹⁷ and Werner and Böhm.¹⁸ Each digestion step was optimized separately. The AA of the in vitro digestion treatment (enzyme and pH adjustment alone) was minimal (0.12 mmol TE/100 g).

Salivary Step. Two hundred and fifty milligrams of aqueous sample extracts were crushed lightly and dissolved for 2 min in 4 mL of α -amylase in PBS solution (160 units/mL; adjusted to pH 6.5) by using a preheated pestle and mortar at 37 °C.

Gastric Step. Immediately after the salivary step, the mixture was acidified to pH 3.0 (with 0.1 M HCl) and incubated with pepsin solution in 0.1 M HCl for 60 min at 37 °C at ~190 strokes/min.

Duodenal Step. Following the gastric simulation step, the mixture was raised to pH 6.5–7 with 1 M NaHCO₃ and incubated with pancreatin and bile extract in 0.1 M NaHCO₃ for 120 min at 37 °C in a shaking water bath at 190 strokes/min. The in vitro digested mixture was centrifuged at 2060g for 5 min at 4 °C, filtered through filter paper (Whatman grade 1) and a 0.2 μ m pore size nylon filter, and adjusted to pH 5.5–6.0 with 0.1 M HCl. Digested samples were stored at –20 °C until analysis for no longer than 1 week.

Preparation of Nondigested (Control) Sample Extracts. Nondigested controls were prepared by extracting 250 mg per sample in water to a concentration matching the concentration of the in vitro digested sample mixtures.

Antioxidant Assays. Except for the lipophilic ORAC assay, the 25 nondigested DS were extracted in a 10-fold volume of methanol to weight (250 mg DS/2.5 mL). Samples were vortexed (10 min), sonicated (10 min), and centrifuged (10 min at 2000g). Supernatants were diluted with aqueous buffers depending on the assay. Sonication had no significant effect on the AA. For the digested and nondigested (control) DS, extraction was performed with water as mentioned above.

Trolox Equivalent Antioxidative Capacity (TEAC). The TEAC was determined by measuring the ability to scavenge ABTS radical cations produced by oxidation of ABTS with manganese dioxide. The assay was performed as previously reported.¹⁹ Briefly, ABTS radical cations were prepared by adding solid manganese dioxide (80 mg) to a 5 mM aqueous stock solution of ABTS⁺ (20 mL using a 75 mM sodium/potassium (Na/K) buffer of pH 7). Trolox, a water-soluble analogue of vitamin E, was used as an antioxidant standard. A standard calibration curve was constructed for Trolox at 0–0.3 mM concentrations. DS extracts (250 mg/2.5 mL of MeOH) were diluted in Na/K buffer. Diluted samples were mixed with 200 μ L of ABTS⁺ radical cation solution in 96-well plates, and absorbance was read (at 750 nm) after 30 min in a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). TEAC values were calculated from the Trolox standard curve and expressed as Trolox equivalents (mmol TE/100 g). All samples were extracted in duplicate, and the TEAC assay was performed in triplicate for each extract.

Total Oxygen Radical Absorbance Capacity (ORAC). The ORAC assay was conducted as previously described.²⁰ For the hydrophilic ORAC a mixture of 200 μ L of fluorescein sodium salt (0.1 μ M) was used as the target of free radical attack, and 75 μ L of 2,2'-azobis(amidinopropane) dihydrochloride (AAPH) (63.4 mM) was used as a peroxy radical generator at 37 °C combined with 20 μ L of each sample extract. DS extracts (250 mg/2.5 mL of MeOH) were diluted in Na/K buffer. Trolox standards ranged from 5 to 100 μ M. For the lipophilic ORAC DS were extracted twice using hexane. The hexane extracts were combined and evaporated. The dried hexane extract was dissolved in 250 μ L of acetone and diluted with 750 μ L of 7% randomly methylated β -cyclodextrin (RMCD) solution (50% acetone/50% water, v/v). Extracts were further diluted in 7% RMCD. Trolox standard was also diluted in 7% RMCD. AAPH concentration was adjusted to provide a decay curve with no more than 5% of antioxidant activity remaining at the end of the reaction. The decrease in fluorescence was determined at excitation of 535 nm and emission of 595 nm every 2 min for 60 min in a SpectraMax GeminiEM plate reader (Molecular Devices). The ORAC value was evaluated as the area under curve (AUC) and calculated by taking into account the Trolox reading using the following equation: $(AUC_{\text{sample}} - AUC_{\text{buffer}})/$

$(AUC_{\text{Trolox}} - AUC_{\text{buffer}}) \times \text{dilution factor of sample} \times \text{initial Trolox concentration (mM)}$. For each sample, four serial dilutions in phosphate buffer (pH 7.4) were measured. The optimum concentration was used for further analyses in four to six replicates.

Free Radical Scavenging Capacity. The free radical scavenging capacity was analyzed by the DPPH assay.¹⁹ DPPH is a stable organic nitrogen radical with purple color. Upon reduction by an antioxidant, the solution color fades and the reaction can be monitored by a spectrophotometer at 517 nm. The decrease in absorbance is proportional to the antioxidant capacity and can be measured in comparison to Trolox as standard. All samples and standard were diluted in methanol. Twenty microliter aliquots from the test compounds were mixed with 200 μ L of DPPH (0.6 mM) in methanol, and the change in optical density (517 nm) was monitored after 30 min using a VersaMax plate reader (Molecular Devices).

Ferric Reducing Antioxidant Power Assay. The FRAP assay was performed using an established standardized method described by Benzie and Strain.²¹ Reaction mixtures were prepared by combining 10 mM 2,4,6-tri(2-pyridyl-s-triazine) (TPTZ), 20 mM ferric chloride, and 30 mM (pH 3.6) sodium acetate buffer in a 1:1:10 ratio. Trolox was used as standard in a range from 0 to 0.1 mM. Samples extracted in a 10-fold volume of methanol (w/v) were diluted in acetate buffer. A 200 μ L reaction solution was warmed to 37 °C for 10 min prior to the addition of 20 μ L of diluted sample extracts. Sample absorbance was read at 593 nm in a VersaMax plate reader (Molecular Devices). Results were compared to the Trolox standard curve and expressed as TE.

Chemical Analysis. Chemical markers were analyzed by high-performance liquid chromatography (HPLC) using a Waters Alliance 2690/2695 HPLC system (Waters, Milford, MA, USA) with a 996 PDA detector. GT chemical markers were extracted with a 75% MeOH/25% H₂O mixture and separated using a Phenomenex Synergi 4 μ Hydro-RP column (150 \times 4.6 mm, 80A) (Phenomenex, Torrance, CA, USA) with a gradient mobile phase of 0.25% H₃PO₄ in water (solvent A) and 0.1% H₃PO₄ in acetonitrile/MeOH (1:1, v/v) + 1.0% THF (solvent B). Solvent composition starting at $T = 0$ min with 95% solvent A and 5% solvent B changed to 20% solvent A and 80% B at $T = 50$ min and back to the initial mixture at $T = 51$ –60 min. All standards were purchased from Sigma-Aldrich Co. or ChromaDex (Irvine, CA, USA). The pomegranate DS were homogenized in dimethyl sulfoxide, sonicated, and vortexed for 20 min each, diluted with methanol/water (1:1, v/v), centrifuged for 5 min at 8000g, and separated on a Zorbax SB C₁₈ column (5 μ m 4.6 \times 250 mm) (Agilent, Santa Clara, CA, USA) using a gradient of 0.1% H₃PO₄ in water (solvent A) and acetonitrile (solvent B). Solvent mixture changed from $T = 0$ min at 95% solvent A and 5% solvent B to 60% solvent A and 40% solvent B at 30 min. Resveratrol was quantified in a methanol extract using an Agilent Poroshell120 SB-C18 column (2.7 μ m 100 \times 4.6 mm) with a gradient mobile phase of 0.4% H₃PO₄ in water (solvent A) and acetonitrile (solvent B). The solvent mixture was changed from $T = 0$ min at 80% solvent A and 20% solvent B to 40% solvent A and 60% solvent B at 8 min and returning to initial solvent mixture at 9–13 min.

Determination of Total Phenolics. The colorimetric method by Singleton et al.²² was used to determine the total phenol content. Gallic acid was used to prepare a standard curve, and final values are expressed in gallic acid equivalents (GAE). Diluted DS extracts were incubated with Folin–Ciocalteu phenol reagent (Sigma-Aldrich Co.). The color was developed by adding sodium carbonate (20%) and absorbance measured at 755 nm.

Statistical Analysis. Values in the results are means (95% CI), unless otherwise specified. With regard to nondigested antioxidant activity of all 25 samples, data were analyzed separately for each antioxidant assay method using one-way ANOVA in IBM SPSS Statistics (version 21.0; IBM Corp., Armonk, NY, USA), with type of DS as a between-group factor. Multiple-comparisons testing was adjusted using Tukey's honestly significant difference test when there was a significant group effect. Pearson's correlation coefficient was used to investigate the pairwise associations between variables, unless

Table 2. Antioxidant Capacity of 25 Non-digested Antioxidant DS Available in the United States^a

	TEAC ^b in MeOH ^c mmol TE/100 g	FRAP ^b in MeOH mmol TE/100 g	DPPH ^b in MeOH mmol TE/100 g	ORAC- hydro ^b mmol TE/100 g	ORAC- lipo mmol TE/100 g	sum of methods ^b mmol TE/100 g
PM1	803.9	578.8	536.2	452.6	0.103	2371.6
PM2	70.2	34.6	27.7	6.9	0.083	139.5
PM3	519.0	400.3	364.5	301.9	1.205	1586.9
PM4	843.0	633.3	693.1	417.2	1.152	2647.5
PM5	1141.7	853.2	780.6	471.3	0.108	3246.8
meanH ^d ± SD	826.9 ± 254.7d	616.4 ± 186.6d	593.6 ± 183.1d	410.8 ± 75.9	0.6 ± 0.6	2448.3 ± 684.3d
meanL ^d ± SD	675.6 ± 404.0	500.1 ± 306.3	480.4 ± 298.7	330.0 ± 192.2	0.5 ± 0.6	1986.5 ± 1190.5
GT1	207.7	106.8	119.5	159.2	0.214	593.4
GT2	381.2	168.4	204.5	242.7	0.032	996.9
GT3	274.6	128.9	166.7	240.0	0.226	810.4
GT4	1054.5	583.0	574.4	673.8	0.343	2886.0
mean ± SD	479.5 ± 389.9	246.8 ± 225.6e	266.3 ± 208.3e	328.9 ± 233.2	0.2 ± 0.1	1321.7 ± 1055.9
Res1	423.5	100.8	63.6	632.9	16.95	1237.8
Res2	916.6	254.1	159.6	1259.5	1.352	2591.2
Res3	435.5	155.3	148.2	486.7	1.094	1226.8
mean ± SD	591.9 ± 281.3	170.0 ± 77.7e	123.8 ± 52.4e	793.0 ± 410.5d	6.5 ± 9.1	1685.3 ± 784.6
GS1	156.2	76.0	112.2	167.0	1.827	513.2
GS2	204.6	101.0	131.2	285.8	0.176	722.7
GS3	592.3	335.3	402.7	333.4	0.009	1663.6
mean ± SD	317.7 ± 239.0	170.8 ± 143.0e	215.4 ± 162.5e	262.1 ± 85.7e	0.7 ± 1.0	966.5 ± 612.7
MT1	211.7	42.5	27.0	54.2	1.411	336.8
MT2	228.2	42.0	49.5	63.8	0.069	383.6
MT3	313.4	67.0	49.8	75.3	0.314	505.9
MT4	244.2	46.7	54.2	71.7	0.095	416.9
mean ± SD	249.4 ± 44.7e	49.6 ± 11.8e	45.1 ± 12.3e	66.3 ± 9.4	0.5 ± 0.6	410.8 ± 71.4e
Acai1	0.5	0.7	0.3	1.2	0.810	3.6
Acai2	4.4	2.3	n.d.	3.9	0.352	10.9
mean ± SD	2.5 ± 2.7e	1.5 ± 1.1e		2.6 ± 1.9e	0.6 ± 0.3	7.3 ± 5.2e
Acai+GT	181.0	94.0	117.9	209.5	14.3	616.7
Goji1	2.2	1.4	2.6	3.2	2.4	11.8
Goji2	0.4	0.1	0.2	0.8	0.008	1.6
Goji3	5.5	1.3	1.4	15.5	0.04	23.7
mean ± SD	2.7 ± 2.6e	0.9 ± 0.7e	1.4 ± 1.2e	6.5 ± 7.9e	0.8 ± 1.4	12.4 ± 11.1e

^aDS, dietary supplements; TEAC, Trolox equivalent antioxidant capacity; DPPH, diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; ORAC, oxygen radical absorbing capacity; TE, Trolox equivalents; PM, pomegranate; GT, green tea; Res, resveratrol; GS, grape seed; MT, milk thistle. Mean values within a column with unlike letters were significantly different ($P < 0.05$; Tukey's multiple-comparison test). ^bOne-way ANOVA of the absolute values in antioxidant activity for DS category effect; $P < 0.01$. ^cExcept for acai and goji, which do not mix well with MeOH and were extracted in water. ^dPM2 is excluded from meanH (higher mean) and is included in meanL (lower mean).

Spearman's rho was used where specified. Differences were considered significant at $P < 0.05$.

RESULTS

Antioxidant Activity of 25 Nondigested DS by Different Assays. A total of 25 of the most popular DS categories sold in the United States were tested for their antioxidant activity by TEAC, DPPH, ORAC, and FRAP assays; methanol was used as the extraction solvent except for the lipophilic ORAC, in which hexane was used (Table 2). One-way ANOVA of the absolute values in antioxidant activity demonstrated a significant DS type effect for TEAC ($P =$

0.004), FRAP ($P = 0.000$), DPPH ($P = 0.000$), OPAC-hydro ($P = 0.001$), sum of methods ($P = 0.001$), but not for ORAC-lipo (NS). The AA varied depending on the solubility in water or organic solvents.

TEAC. Pairwise testing showed significantly higher TEAC values for pomegranate versus milk thistle ($P < 0.05$), acai ($P < 0.025$), and goji ($P < 0.01$); no other differences between types of DS reached statistical significance.

FRAP. FRAP values were higher for pomegranate versus green tea ($P < 0.025$), grape seed ($P = 0.01$), milk thistle ($P < 0.001$), resveratrol ($P = 0.01$), acai ($P < 0.01$), and goji ($P <$

Table 3. Correlation Matrix of the Different Antioxidant Assays^a

		TEAC	FRAP	DPPH	ORAC-hydro	ORAC-lipo
TEAC	Pearson correlation	1	0.918^b	0.879^b	0.788^b	-0.055
	signif (2-tailed)		0.000	0.000	0.000	0.793
	N	25	25	24	25	25
FRAP	Pearson correlation	0.918^b	1	0.987^b	0.545^b	-0.131
	signif (2-tailed)	0.000		0.000	0.005	0.533
	N	25	25	24	25	25
DPPH	Pearson correlation	0.879^b	0.987^b	1	0.465^c	-0.163
	signif (2-tailed)	0.000	0.000		0.022	0.446
	N	24	24	24	24	24
ORAC-hydro	Pearson correlation	0.788^b	0.545^b	0.465^c	1	0.198
	signif (2-tailed)	0.000	0.005	0.022		0.343
	N	25	25	24	25	25
ORAC-lipo	Pearson correlation	-0.055	-0.131	-0.163	0.198	1
	signif (2-tailed)	0.793	0.533	0.446	0.343	
	N	25	25	24	25	25

^aTEAC, Trolox equivalent antioxidant capacity; DPPH, diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; ORAC, oxygen radical absorbing capacity. ^bCorrelation is significant at $P < 0.01$. ^cCorrelation is significant at $P < 0.05$.

Table 4. Chemical Compositions (Percent) of Green Tea (GT), Pomegranate (PM), and Resveratrol (Res) DS^a

content	(A) Green Tea				
	GT1	GT2	GT3	GT4	
theobromine	0.1 ± 0.002	0.08 ± 0.005	0.08 ± 0.001	0.11 ± 0.001	
EGC	1.6 ± 0.05	2.4 ± 0.123	1.5 ± 0.023	2.4 ± 0.004	
(±)-catechin	0.4 ± 0.03	0.2 ± 0.007	0.66 ± 0.01	0.88 ± 0.002	
caffeine	2.7 ± 0.08	3.8 ± 0.05	3.1 ± 0.02	1.26 ± 0.05	
EC	0.5 ± 0.02	0.67 ± 0.04	0.64 ± 0.02	2.25 ± 0.014	
EGCG	3.35 ± 0.2	6.5 ± 0.38	3.98 ± 0.04	27.16 ± 0.18	
ECG	2.05 ± 0.1	4.27 ± 0.25	3.78 ± 0.01	13.98 ± 0.006	
sum of catechins	7.9	14.0	11.1	46.7	
content	(B) Pomegranate				
	PM1	PM2	PM3	PM4	PM5
punicalagins A and B	4.4 ± 0.04	0	11.9 ± 0.2	22.8 ± 0.2	28.2 ± 0.5
ellagic acid	4.3 ± 0.07	24.6 ± 0.20	0.9 ± 0	21.5 ± 0.14	1.7 ± 0.04
content	(C) Resveratrol				
	Res1	Res2	Res3		
quercetin	0.15 ± 0.0	18.52 ± 0.12	0.03 ± 0.002		
resveratrol	18.2 ± 0.05	32.1 ± 0.3	10.9 ± 0.05		

^aDS, dietary supplements; EGC, (-)-epigallocatechin; EC, (-)-epicatechin; EGCG, (-)-epigallocatechin gallate; ECG, (-)-epicatechin gallate.

0.001); no other differences between other DS types reached statistical significance.

DPPH. Pairwise testing demonstrated higher DPPH values for pomegranate versus green tea ($P < 0.05$), grape seed ($P < 0.05$), milk thistle ($P = 0.001$), resveratrol ($P < 0.01$), and goji ($P = 0.001$); no other differences between other DS types reached statistical significance (acai not included in the model due to lack of sample power).

ORAC-hydro. Contrary to the TEAC, FRAP, and DPPH, ORAC-hydro values were significantly higher only for resveratrol compared to grape seed ($P < 0.05$), acai ($P < 0.01$), goji ($P = 0.001$), and green tea ($P = 0.052$).

ORAC-lipo. The lipophilic ORAC evaluates the AA of the lipophilic antioxidants. The contribution of the AA measured by the lipophilic ORAC is minor in the supplements with high

polyphenolic content. However, for goji juice (Goji1), the lipophilic ORAC contributed considerable AA to the total activity, whereas the goji extracts (Goji2 and Goji3) lost most of the lipophilic AA (Table 2).

Sum of Methods. For the sum of all methods that measure the AA, results were highly correlated to TEAC values; we found that the antioxidant capacity is highest in pomegranate and decreasing in the following order: pomegranate \gg resveratrol $>$ green tea $>$ grape seed $>$ milk thistle \gg goji $>$ acai, with significance reached for pomegranate versus milk thistle, acai, and goji ($P < 0.01$).

In addition, TEAC was highly correlated with FRAP, DPPH, and ORAC-hydro ($r = 0.79$ – 0.92 , $P < 0.001$), and FRAP and DPPH were highly correlated with each other ($r = 0.99$, $P < 0.001$) and moderately correlated with ORAC-hydro ($r =$

0.47–0.55, $P < 0.025$). The correlation matrix is depicted in Table 3.

Variation of AA in Each Category. Extensive differences in AA were observed among the DS in each category. For example, PM2 exhibited significantly lower AA compare to the other four pomgranate DS; GT4, Res2, and GS3 exhibited significantly higher AA compared to other DS in each category. This was closely correlated to the chemical composition of marker compounds.

Chemical Analyses and Associations with Antioxidant Activity. DS from pomegranate, green tea, and resveratrol/ quercetin were analyzed using HPLC with PDA detection, and the concentration of known chemical markers was quantified before and after digestion (Tables 4 and 5). Total phenolic content was determined for the DS with high polyphenol content before and after digestion using the GAE assay (Table 6).

Table 5. Change in Content of Chemical Marker Compounds in Green Tea, Pomegranate, and Resveratrol DS during in Vitro Digestion^a

dietary supplement	marker chemical	% of control
PM5	punicalagins A and B	88.5
	ellagic acid	208.7
GT4	theobromine	70.2
	EGC	51.0
	(±)-catechin	81.5
	caffeine	87.2
	EC	78.4
	EGCG	63.0
	ECG	73.3
	sum of catechins	66.5
Res2	resveratrol	239.8

^aPM, pomegranate; GT, green tea; EGC, (–)-epigallocatechin; EC, (–)-epicatechin; EGCG, (–)-epigallocatechin gallate; ECG, (–)-epicatechin gallate; Res, resveratrol.

In pomegranate supplements, punicalagins A and B and ellagic acid were quantified. Pomegranate supplements with the highest content of punicalagins A and B (PM4 and PM5) showed high antioxidant activity, whereas pomegranate supplements with high ellagic acid showed the lowest antioxidant activity (PM2); a highly significant correlation coefficient was reported between pomegranate antioxidant activity by TEAC, FRAP, DPPH, or sum of methods and punicalagin A and B content (Spearman's rho = 0.90, $P < 0.05$). Although PM1 had low content of punicalagins A and B and ellagic acid, its antioxidant activity was relatively high. It appears that other polyphenols or ellagitannins of larger molecular weights may have contributed considerably to the AA of PM1, as indicated by the high content of total phenolics, expressed as GAE, in PM1. In fact, the AA was increased by 50% for PM1 after digestion compared to nondigested controls (PM1-digested, 667.1 ± 23.1 mmol TE/100 g; PM1-control, 420.6 ± 76.2 mmol TE/100 g).

Among the GT supplements, the ECG, EC, and EGCG contents were highly correlated ($r = >0.99$; $P < 0.01$) with the AA measured by TEAC, FRAP, DPPH, and ORAC-hydro, with the correlation coefficient between sum of catechins and sum of methods reaching $r = 1.0$ ($P < 0.001$). GT4 reported the highest catechin concentration and highest AA. However, after digestion, the catechin content of known chemical markers

(ECG, EC, CG, EGC, EGCG, and GCG) was decreased by 90%, whereas the AA decreased by only 22%.

The resveratrol DS contained mixtures of resveratrol and quercetin, with quercetin highly correlating with TEAC ($r = 1.0$, $P < 0.025$) and sum of methods ($r = 1.0$, $P = 0.001$) and with resveratrol showing similar associations but without reaching significance due to the small sample size ($r > 0.93$, $P = 0.22$, $n = 3$). Within samples tested, Res2 showed the highest resveratrol/quercetin content and highest AA. Total phenolic content highly correlated with TEAC for both digested and nondigested (control) DS samples ($r = 0.9956$, $P < 0.01$).

Antioxidant Activity of Digested DS Samples. On the basis of the highest AA in the nondigested samples, one DS from each category was selected for in vitro digestion. The AA measured in the digested samples is summarized in Table 6. The order of antioxidant potency of the in vitro digested DS was as follows: PM5 > GT4 > GS3 \gg Res2, MT3 > goji3, acai2 extracts. PM5 reported the highest TEAC values, 427 times higher than that of acai, the DS that exhibited the lowest TEAC values: 1109.4 ± 8.2 mmol TE/100 g compared to 2.6 ± 0.1 mmol TE/100 g, respectively. TEAC of in vitro digested GT4 and GS3 was 73 and 46.5% that of PM5 and 16.6 and 10.5 times greater than that of Res2, respectively.

Digested versus Nondigested (Control) DS Samples. The digestion was performed with water extracts of all DS and was compared to nondigested DS samples extracted in water (Table 6). Other than for GT4 and GS3, which showed 21.5 and 8.1% lower TEAC values after in vitro digestion compared to the nondigested samples extracted in H₂O, the other DS samples demonstrated either similar or higher TEAC values after in vitro digestion (Table 6). For resveratrol and milk thistle the AA was significantly lower in the digested sample compared to the nondigested sample extracted in methanol (Table 3) because their solubility is higher in methanol compared with water. For goji and acai the AA was very low in the digested and nondigested samples extracted in water or methanol.

DISCUSSION

To our knowledge, this is the first study to comprehensively evaluate and compare the antioxidant potency of commercially available polyphenol-rich antioxidant DS, including extracts from pomegranate, green tea, grape seed, resveratrol, milk thistle, and acai, and goji berry, utilizing different antioxidant assays before and after in vitro digestion. In the present study, pomegranate DS expressed the most potent antioxidant capacity, followed by resveratrol, green tea, grape seed, and milk thistle extracts, whereas acai and goji berry demonstrated negligible antioxidant properties across all four antioxidant assays and in the in vitro digestion model. The AA of resveratrol extract and milk thistle was influenced by the solvent used for extraction, with 10–20-fold greater values comparing the methanol to water extract. Hence, it appears that both resveratrol and milk thistle DS may efficiently express their AA when ingested in ethanol (alcoholic) solutions. There is earlier evidence showing that the solubility of *trans*-resveratrol in ethanol plus water mixtures increases with increasing mole fraction of ethanol,²³ which together with the present data might partly explain red wine's antioxidant properties. Previous studies on milk thistle evaluated its AA mainly in aqueous extracts^{24,25} and have demonstrated even lower values for TEAC, with one study²⁵ reporting 133.4 μ g TE/g of milk

Table 6. Gallic Acid Equivalents (GAE) and Antioxidant Activity by TEAC Method of in Vitro Digested and Nondigested (Control) DS^a

	GAE (mg/L)	TEAC (mmol TE/100 g)	digested (% of control H ₂ O)
PMS			
digested (H ₂ O)	16924 ± 489	1109.4 ± 8.2	101.7
control (H ₂ O)	17419 ± 1027	1090.5 ± 24.6	
GT4			
digested (H ₂ O)	11075 ± 172	811.7 ± 39.1	78.5
control (H ₂ O)	14779 ± 959	1034.1 ± 15.8	
GS3			
digested (H ₂ O)		515.9 ± 12.7	91.9
control (H ₂ O)		561.2 ± 70.2	
Res2			
digested (H ₂ O)	771 ± 2	49.0 ± 6.2	124.8
control (H ₂ O)	552 ± 14	39.2 ± 4.0	
MT3			
digested (H ₂ O)		35.8 ± 0.8	720.1
control (H ₂ O)		5.0 ± 0.01	
Goji3			
digested (H ₂ O)		10.8 ± 0.5	175.6
control (H ₂ O)		6.1 ± 0.4	
Acai2			
digested (H ₂ O)		2.6 ± 0.05	124.5
control (H ₂ O)		2.1 ± 0.01	

^aDS, dietary supplements; TEAC, Trolox equivalent antioxidant capacity; TE, Trolox equivalents; PM, pomegranate; GT, green tea; GS, grape seed; Res, resveratrol; MT, milk thistle.

thistle powder compared to 5 mmol TE/100 g = 12.5 mg TE/g of our aqueous milk thistle extract sample (Table 6). A similar ranking of the AA of beverages from previous research from our laboratory also showed the highest AA for pomegranate > red wine > grape juice.¹⁹ Tea did not show considerable AA because bottled tea beverages were used.¹⁹

Significant variation in AA between DS from the same category corresponding to variation in the chemical composition was observed, although the DS, according to the label information, are supposed to provide the same health benefits. The AA of pomegranate DS was closely associated with the chemical composition. Except for PM2, all other pomegranate DS showed the highest AA. The AA of PM2 was very low compared to the other pomegranate supplements because it mainly contains ellagic acid and no punicalagins A and B. As previously described,²⁶ the official marker compound for pomegranate supplements is ellagic acid, whereas authentic pomegranate has only small amounts of ellagic acid. Whether ellagic acid from other sources is added to these supplements or the increase in ellagic acid occurred as the result of manufacturing was not determined. However, high amounts of ellagic acid only resulted in lower AA compared to supplements including punicalagins. Differential response of two pomegranate DS (PM1 and PMS) to in vitro digestion with α -amylase, pepsin, pancreatin, and bile salts was observed. Although PMS showed no change of AA, the AA was increased by 50% for PM1 compared to nondigested controls. It appears that because PM1 contains relatively low concentrations of punicalagins A and B and ellagic acid, its AA is based on larger ellagitannins, which, during the in vitro digestion process, are further dissolved or converted to smaller compounds with higher antioxidant activity. Similar results have been found in another study where the AA of pomegranate juice increased significantly after in vitro digestion.²⁷ In vivo, the hydrolysis of pomegranate ellagitannins to ellagic acid and further metabo-

lism by the gut microbiota to yield urolithins have been documented.^{28–30} These metabolites in turn exhibit AA.³¹

In the current study, the antioxidant supplements with the lowest AA, and of minor significance, were acai and goji berries. Results from other published in vitro and in vivo studies testing the antioxidant effects of acai juice/pulp or goji berry fruit demonstrated strong variability in AA due to differences in sourcing and processing of the material. Freeze-dried acai pulp/skin powder demonstrated 1.03 mmol TE/g by the ORAC assay.³² Enhanced AA in human plasma has been shown for acai pulp and acai juice after a single ingestion in one study utilizing the ORAC assay.³³ In vitro analyses of goji fruit extracts have captured its antioxidant properties, including inhibition of DPPH[•] radical-scavenging activity.^{14,15,34} A study by Luo et al.³⁵ reported a stronger in vitro AA by TEAC and ORAC assays of goji fruit water, methanol extracts, and crude polysaccharide extracts compared to purified polysaccharide fractions. In the study by Luo, goji demonstrated levels of TEAC values of maximal 0.5 mmol TE/100 g, which are lower than the AA demonstrated by our goji samples.³⁵ Most likely the differences in antioxidant activity in the present study and earlier investigations of acai and goji berries are caused by losses of AA during processing of the berry pulp during manufacturing of the supplements. The main antioxidant compounds in acai and goji berries are anthocyanins, carotenoids, and polysaccharides, respectively. Anthocyanins are prone to degradation during processing and storage (pH, temperature, light, oxygen, enzymes, presence of ascorbic acid, sugars, sulfur dioxide or sulfite salts, metal ions, and copigments)³⁶ and were not likely to contribute to antioxidant activities, which were very small in any case. In the lipophilic ORAC test, the goji berries demonstrated high variability, with the goji juice (Goji1) exhibiting somewhat higher lipophilic AA compared to the goji extracts (Goji2 and Goji3), suggesting that the goji juice contained some carotenoids.

In the present study, in vitro simulation of gastrointestinal digestion did not adversely affect the antioxidant capacity of any of the DS extracts, other than for green tea, which lost 21.5% of AA during digestion. Similarly, earlier work on the impact of in vitro digestion on commercially available tea juices (longjing green tea, jasmine, and oolong) demonstrated a significant decrease of AA as well as total polyphenol content.³⁷ In the presented study the tea polyphenol content decreased by only 10%. Possibly the exposure to alkaline pH transformed EGCG and EGC to hetero- and homodimers such as theasinensin, which most likely retained the antioxidant activity.³⁹ A small decrease in AA was also observed for grape seed extracts (8.1%). However, a study by Janisch demonstrated an increase in release of flavans and procyanidins from grape seed extract with good scavenging potential against reactive oxygen species under conditions of in vitro digestion.³⁸ In addition to potential changes through the digestive process, possible interactions of food ingested concurrent with the DS have been reported⁴⁰ and more detailed future evaluations are warranted.

In agreement with our previous study on polyphenol-rich antioxidant beverages, the order of antioxidant capacity correlated well between the different methods with the exception of the ORAC method. The ORAC assay principle is based on a temperature-dependent free radical generator reaction and protection from oxidation over a 60 min period. Although the ORAC method is the most commonly used assay by food manufacturers,¹⁹ our laboratory has reported poor reproducibility of this method due to temperature variation in commonly used plate readers (unpublished data). As we concluded in our earlier paper, it is important to run multiple antioxidant methods rather than just the ORAC method to get a better estimate of antioxidant capacity and to substantiate in vitro results with clinical studies. The TEAC assay correlated best to the sum of the assays and was the most reproducible. Using the DPPH assay all dilutions are performed using methanol instead of buffer, which lowers the reproducibility.

In conclusion, our results demonstrate that except for green tea and grape seed extracts no significant loss of antioxidant activity was observed during in vitro digestion.

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Author Contributions

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Notes

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ABBREVIATIONS USED

DPPH, diphenyl-1-picrylhydrazyl; DS, dietary supplements; EC, (–)-epicatechin; ECG, (–)-epicatechin gallate; EGC, (–)-epigallocatechin; EGCG, (–)-epigallocatechin gallate;

GAE, gallic acid equivalents; FRAP, ferric reducing antioxidant power; NHANES, National Health and Nutrition Examination Survey; ORAC, oxygen radical absorbing capacity; PM, pomegranate; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity

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